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Specific expression of human endothelin-2 (ET-2) gene in a renal adenocarcinoma cell line

Molecular cloning of cDNA encoding the precursor of ET-2 and its characterization

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Analysis of culture medium of human renal adenocarcinoma cells ACHN by RP-HPLC suggested that the cells specifically secreted human endothelin-2 (ET-2). cDNAs encoding human ET-2 precursor were cloned from a cDNA library constructed with mRNA derived from the ACHN cells, and the nucleotide and deduced amino acid sequences were determined. The ET-2 cDNA was revealed to contain 1.3 kb and encode prepro-ET-2 protein consisting of 178 amino acid residues. The ET-like sequence found in the prepro-ET-1 and -ET-3 was conserved in this prepro-ET-2. The Northern blot analysis of mRNA suggested that the transcript of ET-2 gene was 1.4 kb. This is the first direct evidence that human ET-2 gene was expressed specifically in tumor cells.

Human endothelin-2; cDNA cloning; Gene expression; ACHN cell

1. INTRODUCTION

In an earlier work a novel vasoconstrictor peptide, endothelin, was purified from a culture medium of porcine aortic endothelial cells, and its structure and the nucleotide sequences of the cloned cDNA encoding the precursor were determined [1]. Also, human cDNA encoding prepro-endothelin has been cloned, and the amino acid sequences of human and porcine endothelin have been identified [2]. Recently, human endothelin has been suggested to have a gene family comprised of endothelin-1 (ET-1), the first to be recognized, -2 (ET-2) and -3 (ET-3), respectively [3]. Based on the deduced amino acid sequences of the cloned ET-2 and ET-3 gene, ET-2 and ET-3 have been chemically synthesized and the biological activities as the vasoconstrictor of these ETs have been studied. ET-2 was found to have the most potent vasoconstrictive activity among these 3 isopeptides [3]. Direct evidence of the gene expression of ET-3 has recently been revealed by cDNA cloning, Northern blot analysis and the enzyme immunoassay specific for ET-3 [4,5,6]. ET-3 was found to be abundant especially in the rat brain [6].

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Abbreviations: RP-HPLC, reverse phase-high performance liquid chromatography; ET-1, ET-2 and ET-3, endothelin-1,-2 and -3

In contrast to ET-3, little is known about the expression of human ET-2 gene. Very recently cloning of the cDNA encoding precursor of ET-2 has been reported [7].

We previously suggested that ET-2 gene expression might occur in COS-7 cells (SV-40 transformed African green monkey kidney cells). Normal kidney cell lines derived from some species have been found to secrete immunoreactive endothelins [8]. Our studies on animal organs and especially on human cell lines which synthesize ETs revealed that the human renal adenocarcinoma cell line ACHN specifically secretes immunoreactive ET-2, which was characterized by RP-HPLC (to be published). To understand the physiological roles of ET-2 and mechanisms of gene expression, we tried to clone the cDNA encoding the precursor of ET-2 from a cDNA library constructed with mRNA derived from the ACHN cells.

We present here the structure of cDNA encoding the precursor of human ET-2 and direct evidence of ET-2 gene expression specifically in human renal adenocarcinoma cells.

2. MATERIALS AND METHODS

2.1. *Materials*

Dulbecco's minimum essential medium (DMEM) was obtained from Gibco, and fetal calf serum was from Biochem. Guanidine isothiocyanate was obtained from Fluka.

Restriction enzymes, T4 ligase, *Eco*RI linker were obtained from New England Biolabs (Beverly, MA, USA) and Nippon Gene (Tokyo,

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Japan). Exonuclease III and VII were from Takara Shuzo (Kyoto, Japan) and Bethesda Research Labs. [α-^{32}P]dCTP was obtained from Amersham Co.

2.2. *Cell culture*

A human kidney tumor cell line (human renal adenocarcinoma), ACHN, was obtained from the American Type Culture Collection. The cells were cultured in Falcon plastic dishes, 5 cm diameter, with DMEM supplemented with 10% fetal calf serum under the humidified condition of 5% CO_2 and 95% air at 37°C. To prepare messenger RNA (mRNA), ACHN cells were cultured in 150 cm^2 bottles (Falcon) for 3 days.

2.3. *Enzyme immunoassay (EIA)*

Endothelin-1, -2 and -3 specific enzyme immunoassay systems were described previously [9].

2.4. *Reverse phase-high performance liquid chromatography (RP-HPLC) of the culture medium of ACHN cells*

Serum-free culture of the ACHN cells was conducted after the cells became confluent, and for two days the culture medium was harvested for partial characterization of the secreted endothelin with RP-HPLC (TSK-ODS-80 column, 4.6×250 mm, Tosoh, Japan) as described before [8].

2.5. *Northern blot analysis*

Total RNA was extracted from the cultured ACHN cells harvested 3 days after cell seeding using guanidine-isothiocyanate as described [10]. A poly(A)$^+$ RNA was prepared with oligo-dT cellulose spun column obtained from Pharmacia. A 15 μg portion of poly (A)$^+$ RNA was treated with formaldehyde and formamide as described [11], and subjected to electrophoresis on 1% agarose gel.

The separated RNA was transferred to nylon membrane (Pall) followed by heating at 80°C for 3 h. The filter was hybridized with ^{32}P-labelled 99mer oligonucleotides corresponding to the 33 amino acid residues including mature ET-2 predicted from the nucleotide sequences of human ET-2 gene in hybridization buffer containing 50% formamide at 42°C for 20 h. After hybridization, the filter was washed with 2×SSC buffer (saline sodium citrate solution; 1×SSC = sodium chloride, 8.765 g/l, sodium citrate, 4.41 g/l) followed by 0.2×SSC containing 0.1% SDS at 65°C.

2.6 *Construction of cDNA library*

A 5 μg of poly(A)$^+$ RNA prepared from ACHN cells was used to construct a cDNA library with λ phage gt11 using a cDNA cloning kit (Amersham).

2.7. *Cloning and sequence analysis of endothelin-2 cDNA*

The cDNA library containing 1.5×10^6 phage clones was screened with ^{32}P-labelled 150 bp DNA, *NcoI-SphI* human genomic DNA fragment containing whole ET-2 sequences previously cloned (unpublished results) and 99mer oligonucleotides corresponding to the 33 amino acids from His to Val, the 11th amino acid from Cys of N-terminal of ET-2 and the next amino acid of Trp of C-terminal, including whole ET-2 sequences that were predicted from the human genomic ET-2 DNA [3]. Plaque hybridization was conducted at 58°C for 16 h and washed with 2×SSC buffer at 56°C. The insert DNAs of hybridization positive clones, λHET-26, -27, -31, -35, -52 were amplified by polymerase chain reaction (PCR) with λgt11 primers, 5'GGTGGCGACGACTCCTGGAGCCCG and 5'TTGACACCAGACCAACTGGTAAATG (Takara Shuzo, Kyoto) as described [12]. The amplified cDNAs were treated with *Eco*RI and subcloned into the *Eco*RI sites of plasmid pUC 118. After preparation of single stranded DNA with helper phage M13KO7 [13], the nucleotide sequences were determied by the dideoxynucleotide chain termination method [14].

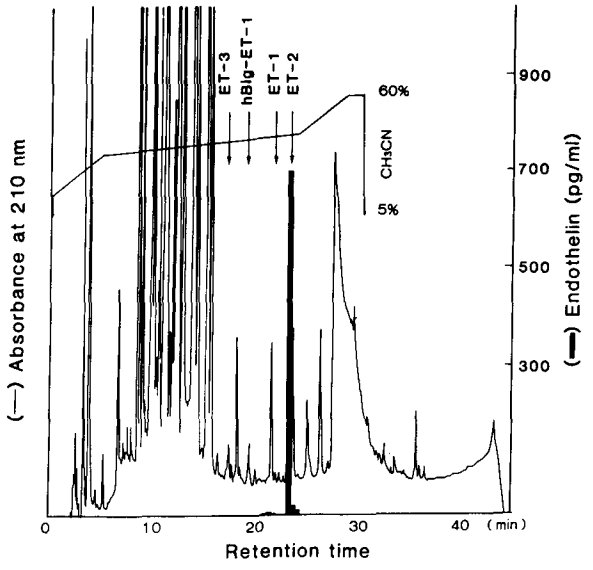

Fig. 1. RP-HPLC profiles of the cultured medium of human renal adenocarcinoma cells (ACHN). Each fraction was concentrated by lyophilization and subjected to EIA specific for ET-1 (ET-2) and ET-3. The arrow shows the position of synthetic endothelin isopeptides eluted on this column.

3. RESULTS

3.1. *Detection of the immunoreactive endothelin in culture medium of ACHN cells*

We had been searching for the cell lines which secrete immunoreactive endothelins and found some, e.g.

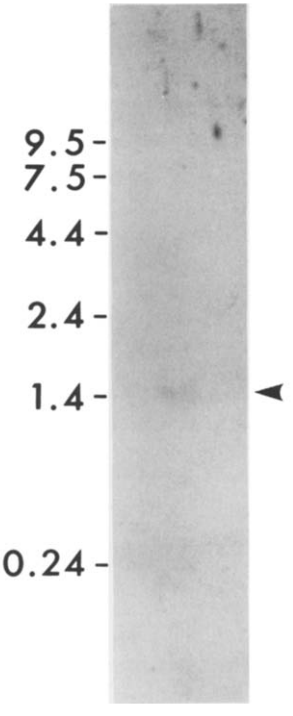

Fig. 2. Northern blot of the poly(A)$^+$ RNA derived from ACHN cells. The molecular size markers are indicated on the left in kilo base.

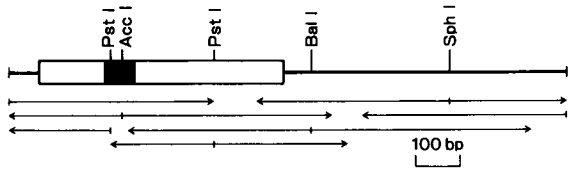

Fig. 3. Restriction enzyme map and sequencing strategy for the cDNA insert in the plasmid pHET-2(K). The coding nucleotide sequences for prepro-ET-2 and mature ET-2 are indicated by open and closed boxes, respectively.

COS-7 [8] and Hep G2 [15]. However, these cell lines secreted a small amount of a mixture of immunoreactive ET-1 and ET-2, which were characterized with RP-HPLC. The human renal adenocarcinoma cell line ACHN studied here was found to secrete a relatively large amount of immunoreactive endothelin into the culture medium. The ACHN cells produced immunoreactive endothelin in parallel with cell growth and the contents reached a plateau at 700–800 pg/ml at 7 days after cell seeding in a culture medium (data not shown). To examine the type of immunoreactive endothelins, the cultured medium of the cells was analyzed by RP-HPLC. The elution profiles of the immunoreactive endothelin are shown in Fig. 1. The position of the eluted immunoreactive material was the same position as that of synthetic ET-2.

3.2. *Detection of transcript related to endothelin-2 gene*

To examine the expression levels and the size of the transcript of ET-2 gene, a 15 μg portion of poly $(A)^+$ RNA derived from ACHN cells was analyzed by Northern blotting with ET-2 specific ^{32}P-labelled 99mer oligonucleotides as described in Section 2. As shown in Fig. 2, a single band was observed near the 1.4 kb size marker supporting the ET-2 gene expression in ACHN cells.

3.3. *Cloning and sequence analysis of cDNAs encoding the precursor of human endothelin-2*

The amplified, *Eco*RI digested cDNA inserts of λHET-26, -27, -31, -35, and -52 were subcloned into the *Eco*RI site of plasmid pUC 118 and named pHET-2(26), -2(27), -2(31), -2(35), and pHET-2(K) (Human Endothelin-2 cDNA from Kidney cell), respec-

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   1                                AGGACGCTGGCAACAGGCACTCCCTGCTCCAGTCCAGCCTGCGCGCTCCACCGCCGCT      58

  59  ATG GTC TCC GTG CCT ACC ACC TGG TGC TCC GTT GCG CTA GCC CTG CTC GTG GCC CTG CAT      118
   1  Met Val Ser Val Pro Thr Thr Trp Cys Ser Val Ala Leu Ala Leu Leu Val Ala Leu His      20

 119  GAA GGG AAG GGC▼CAG GCT GCT GCC ACC CTG GAG CAG CCA GCG TCC TCA TCT CAT GCC CAA      178
  21  Glu Gly Lys Gly Gln Ala Ala Ala Thr Leu Glu Gln Pro Ala Ser Ser Ser His Ala Gln      40

 179  GGC ACC CAC CTT CGG CTT CGC CGT TGC TCC TGC AGC TCC TGG CTC GAC AAG GAG TGC GTC      238
  41  Gly Thr His Leu Arg Leu Arg Arg Cys Ser Cys Ser Ser Trp Leu Asp Lys Glu Cys Val      60

 239  TAC TTC TGC CAC TTG GAC ATC ATC TGG GTG AAC ACT CCT GAA CAG ACA GCT CCT TAC GGC      298
  61  Tyr Phe Cys His Leu Asp Ile Ile Trp Val Asn Thr Pro Glu Gln Thr Ala Pro Tyr Gly      80

 299  CTG GGA AAC CCG CCA AGA CGC CGG CGC CGC TCC CTG CCA AGG CGC TGT CAG TGC TCC AGT      358
  81  Leu Gly Asn Pro Pro Arg Arg Arg Arg Arg Ser Leu Pro Arg Arg Cys Gln Cys Ser Ser      100

 359  GCC AGG GAC CCC GCC TGT GCC ACC TTC TGC CTT CGA AGG CCC TGG ACT GAA GCC GGG GCA      418
 101  Ala Arg Asp Pro Ala Cys Ala Thr Phe Cys Leu Arg Arg Pro Trp Thr Glu Ala Gly Ala      120

 419  GTC CCA AGC CGG AAG TCC CCT GCA GAC GTG TTC CAG ACT GGC AAG ACA GGG GCC ACT ACA      478
 121  Val Pro Ser Arg Lys Ser Pro Ala Asp Val Phe Gln Thr Gly Lys Thr Gly Ala Thr Thr      140

 479  GGA GAG CTT CTC CAA AGG CTG AGG GAC ATT TCC ACA GTC AAG AGC CTC TTT GCC AAG CGA      538
 141  Gly Glu Leu Leu Gln Arg Leu Arg Asp Ile Ser Thr Val Lys Ser Leu Phe Ala Lys Arg      160

 539  CAA CAG GAG GCC ATG CGG GAG CCT CGG TCC ACA CAT TCC AGG TGG AGG AAG AGA TAG TGT      598
 161  Gln Gln Glu Ala Met Arg Glu Pro Arg Ser Thr His Ser Arg Trp Arg Lys Arg ***          178

 599  CGTGAGCTGGAGGAACATTGGGAAGGAAGCCCGCGGGGAGAGAGGAGGAGAGAAGTGGCCAGGGCTTGTGGACTCTCTG      677

 678  CCTGCTTCCTGGACCGGGGCCTTGGTCCCAGACAGCTGGACCCATTTGCCAGGATTGGCACAAGCTCCCTGGTGAGGGA      756

 757  GCCTCGTCCAAGGCAGTTCTGTGTCCTCGCACTGCCCAGGGAAGCCCTCGGCCTCCAGACTGCGGAGCAGCCTCCAGTG      835

 836  CTGGCTGCTGGCCCACAGCTCTGCTGGAAGAACTGCATGGGGAGTACATTCATCTGGAGGCTGCGTCCTGAGGAGTGTC      914

 915  CTGTCTGCTGGGCTACAAACCAGGAGCAACCGTGCAGCCACGAACACGCATGCCTCAGCCAGCCCTGGAGACTGGATGG      993

 994  CTCCCCTGAGGCTGGCATCCTGGCTGGCTGTGTCCTCTCCAGCTTTCCCTCCCCAGAGTTCTTGCACCCTCATTCCCTC     1072

1073  GGGACCCTCCCAGTGAGAAGGGCCTGCTCTGCTTTTCCTGTCTGTATATAACTTATTTGCCCTAAGAACTTTGAGAATC     1151

1152  CCAATTATTTATTTTAATGTATTTTTTAGACCCTCTATTTACCTGCGAACTTGTGTTTATAATAAATGAGGAAACATCA     1230

1231  AAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA              1293
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Fig. 4. Nucleotide sequence and predicted amino acid sequence of the human prepro-endothelin-2 cDNA. The amino acid and nucleotide sequences of mature ET-2 are boxed. Endothelin-like sequence is underlined. The dibasic amino acid pairs are doubly underlined. The probable cleavage site of the signal peptide is indicated by an arrow. The polyadenylation signal sequence is indicated by broken underlining.

tively. The partial restriction enzyme map and sequence strategy of the longest insert cDNA of pHET-2(K) are shown in Fig. 3.

The sequenced nucleotides of ET-2 cDNA of pHET-2(K) was 1218 bp, which encodes the prepro-ET-2 protein consisting of 178 amino acid residues. A putative polyadenylation signal sequence AATAAA was present at the 3'-terminus. The cDNAs of pHET-2(31) and pHET-2(35) were also revealed to have a poly(A) tail. So, the total sequenced nucleotides of ET-2 cDNA were 1293 bp including poly (A) (64 bp) as presented in Fig. 4. Considering the size of ET-2 gene transcript, the molecular size of the cloned ET-2 cDNA was reasonable.

Other clones were also examined to determine the nucleotide sequences, but were found to be incomplete and showed no open reading frame. However, they encode a part of prepro-ET-2 sequence and their nucleotide sequences were conserved. The clones pHET-2(27), pHET-2(31) and pHET-2(35) had deleted 64, 101 and 284 bp from the 5'terminus of the nucleotide sequence of ET-2 cDNA shown in Fig. 4.

The molecular mass of the prepro-ET-2 was calculated to be 19958 Da. The N-terminal 24 amino acids of prepro-ET-2 may be the signal sequence, which can be predicted by the method of von Heijne [16]. The amino acid sequence of the mature ET-2, Cys^{49}–Trp^{69}, is boxed in the figure.

The big form, which was observed in the prepro-ET-1 and -ET-3 as big-ET-1 (1–38 amino acids) and big-ET-3 (1–42 amino acids), was also found in this prepro-ET-2. Big-ET-2 was found to consist of 37 amino acid residues from the amino acid number Cys^{49}–Pro^{85} and might be cleavable at the dibasic amino acid pairs Arg^{47}–Arg^{48} and Arg^{86}–Arg^{87}. Thus big-ET-2 was the smallest of these 3 big-ETs. The endothelin-like (ET-like) sequence, Cys^{96}–Leu^{111} with 4 cysteine residues at the 1, 3, 11, and 15 positions, was also conserved as in the prepro-ET-1 and -ET-3.

In the prepro-ET-2, ET-like peptide, in contrast to that observed in prepro-ET-1 and -ET-3, may be cleaved by peptidase because di-basic amino acid pairs of Arg^{94}–Arg^{95} and Arg^{112}–Arg^{113} are present at the N- and C-terminals of this peptide. The biological function of this peptide remains to be resolved.

4. DISCUSSION

We had shown the structures of the human prepro-ET-1 and -ET-3 before [2,5]. In addition to these structures, the human prepro-ET-2 has been revealed now. Here the structural relationships among prepro-ETs are shown in Fig. 5. The amino acid sequences of the prepro-ET-1 predicted from the nucleotide sequences of cDNA cloned from placenta [2], lung (unpublished data), and umbilical vein endothelial cells [17] were identical and consisted of 212 amino acid residues. However, there are some differences in the prepro-ET-2 and -ET-3. For example, the prepro-ET-3 derived from placenta consisted of 224 amino acid residues [5], but that from the hypothalamus was 238 amino acid residues [4]. In addition to these amino acid differences, 220 bp insertion or deletion was found in these two ET-3 cDNAs. However, the expression of the cloned ET-3 cDNA from human placenta in COS-7 and chinese hamster ovary cells confirmed that the structure of the ET-3 cDNA was reasonable for transcription and translation [5].

The prepro-ET-2 from ACHN cells consisted of 178 amino acid residues, however that from the small intestine had 145 amino acid residues [7]. Some hint as to the reason for these discrepancies might be obtained by examining the structures of the intron and exon in each genomic DNA. The most reasonable explanation may

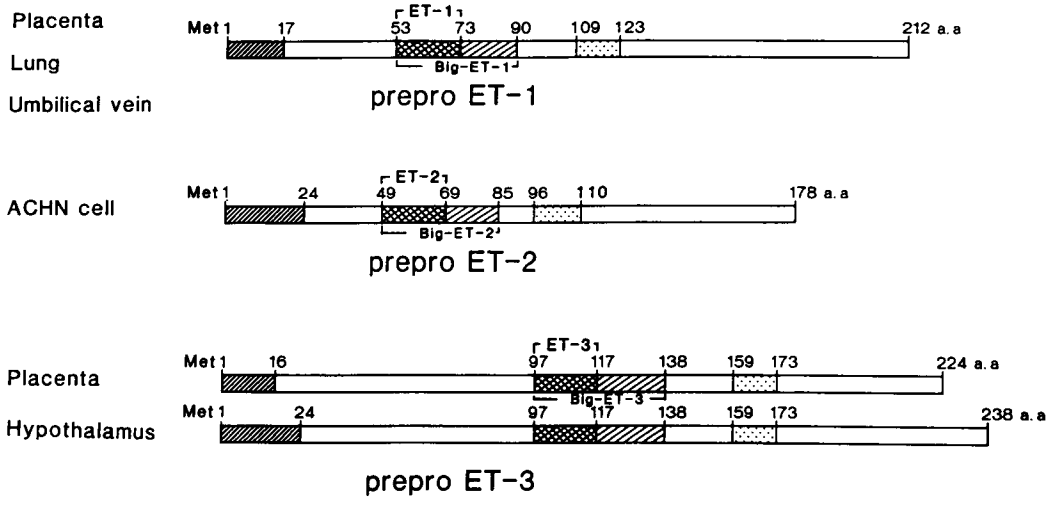

Fig. 5. Structures of prepro-endothelins predicted from their corresponding cDNAs. N-terminal signal sequences are indicated by heavy crosshatching, the mature endothelins by double crosses and the big-ETs by light crosshatching. Endothelin-like sequences are dotted.

be that an alternative splicing occurred. In the case of the ET-2 gene, the ET-2 transcript expressed in small intestine might have no 4th exon present on genomic DNA [7].

The biological activity of endothelin, which was first found to be vasoconstrictor, has been studied extensively in many systems including the vascular one [18]. One of the reported endothelin activities is the stimulation of c-*fos* and c-*myc* gene expression by ET-1 [19]. The tissue distribution of endothelin isopeptides, ET-1, -2 and -3, which may contribute to further understanding of the biological activities has been studied in the rat and the possibility of ET-3 functioning as a brain-gut peptide has been presented [6]. Thus, endothelin isopeptides are revealed to be produced in other non-endothelial cells.

It has been reported that tumor cell lines, including COS-7 cells, HepG2 (hepatocellular carcinoma) and WiDr (colon carcinoma) produce endothelin isopeptides, ET-1 and ET-2 [8,15]. In addition to these cell lines, we have shown that the human renal adenocarcinoma cell line ACHN are typical cells which secrete a large amount of only ET-2 (to be published). In confirming the ET-2 gene expression by cloning the cDNA encoding prepro-ET-2, we have presented here a new view of endothelins and tumors which should lead to better understanding of relationships between endothelins and oncogene expression.

REFERENCES

[1] Yanagisawa, M., Kurihara, H., Kimura, S., Tomobe, Y., Kobayashi, M., Mitsui, Y., Yazaki, Y., Goto, K. and Masaki, T. (1988) Nature 332, 411–415.
[2] Itoh, Y., Yanagisawa, M., Ohkubo, S., Kimura, C., Kosaka, T., Inoue, A., Mitsui, Y., Onda, H., Fujino, M. and Masaki, T. (1988) FEBS Lett. 231, 440–444.
[3] Inoue, A., Yanagisawa, M., Kimura, S. Kasuya, Y., Miyauchi, T., Goto, K. and Masaki, T. (1989) Proc. Natl. Acad. Sci. USA 86, 2863–2867.
[4] Bloch, K.D., Eddy, R.L., Shows, T.B. and Quertermous, T. (1989) J. Biol. Chem. 264, 18156–18161.
[5] Onda, H., Ohkubo, S., Ogi, K., Kosaka, T., Kimura, C., Matsumoto, H., Suzuki, N. and Fujino, M. (1990) FEBS Lett. 261, 327–330.
[6] Matsumoto, H., Suzuki, N., Onda, H. and Fujino, M. (1989) Biochem. Biophys. Res. Commun. 164, 74–80.
[7] Yanagisawa, M., Inoue, A., Imai, Y., Matsushita, Y. and Masaki, T. (1990) Abstract in The Second Endothelin Meeting, January 25–26, held at Tsukuba University, Japan.
[8] Kosaka, T., Suzuki, N., Matsumoto, H., Itoh, Y., Yasuhara, T., Onda, H. and Fujino, M. (1989) FEBS Lett. 238, 249–252.
[9] Suzuki, N., Matsumoto, H., Kitada, C., Masaki, T. and Fujino, M. (1989) J. Immunol. Methods 118, 245–250.
[10] Kaplan, B.B., Bernstein, S.L. and Gillio, A.E. (1979) Biochem. J. 183, 181–184.
[11] Maniatis, T., Fritsch, E.F., Sambrook, J. (1982) Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory, pp. 202–203.
[12] Saiki, R.K., Scarf, S., Faloona, F., Mulli, K.B., Horn, G.T., Erich, H.A. and Arnheim, N. (1985) Science 230, 1350–1354.
[13] Vieira, J. and Messing, J. (1987) Methods Enzymol. 153, 3–11.
[14] Sanger, F., Nicklen, S. and Coulson, A.R. (1977) Proc. Natl. Acad. Sci. USA 74, 5463–5467.
[15] Suzuki, N., Matsumoto, H., Kitada, C., Kimura, S. and Fujino, M. (1989) J. Biochem. 106, 736–741.
[16] von Heijne, G. (1986) Nucleic Acids Res. 134, 4683–4690.
[17] Bloch, K.D., Friedrich, S.P., Lee, M.E., Eddy, R.L., Shows, T.B. and Quertermous, T. (1989) J. Biol. Chem. 1264, 10851–10857.
[18] Yanagisawa, M. and Masaki, T. (1989) Trends Pharmacol. Sci. 10, 374–378.
[19] Komuro, I., Kurihara, H., Sugiyama, T., Takaku, F. and Yazaki, Y. (1988) FEBS Lett. 238, 249–252.